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Shifts in Abundance and Diversity of Mobile Genetic Elements after the Introduction of Diverse Pesticides into an On-Farm Biopurification System over the Course of a Year

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Biopurification systems (BPS) are used on farms to control pollution by treating pesticide-contaminated water. It is assumed that mobile genetic elements (MGEs) carrying genes coding for enzymes involved in degradation might contribute to the degradation of pesticides. Therefore, the composition and shifts of MGEs, in particular, of IncP-1 plasmids carried by BPS bacterial communities exposed to various pesticides, were monitored over the course of an agricultural season. PCR amplification of total community DNA using primers targeting genes specific to different plasmid groups combined with Southern blot hybridization indicated a high abundance of plasmids belonging to IncP-1, IncP-7, IncP-9, IncQ, and IncW, while IncU and IncN plasmids were less abundant or not detected. Furthermore, the integrase genes of class 1 and 2 integrons (*intI1*, *intI2*) and genes encoding resistance to sulfonamides (*sul1*, *sul2*) and streptomycin (*aadA*) were detected and seasonality was revealed. Amplicon pyrosequencing of the IncP-1 *trfA* gene coding for the replication initiation protein revealed high IncP-1 plasmid diversity and an increase in the abundance of IncP-1β and a decrease in the abundance of IncP-1ε over time. The data of the chemical analysis showed increasing concentrations of various pesticides over the course of the agricultural season. As an increase in the relative abundances of bacteria carrying IncP-1β plasmids also occurred, this might point to a role of these plasmids in the degradation of many different pesticides.

Biopurification systems (BPS) are applied on farms to control the environmental pollution caused by the release of water contaminated with pesticides (1). BPS act like biofilters in which the filter matrix (biomix) is composed of a mixture of different adsorbing materials, such as peat, straw, and topsoil (2), and contain microorganisms carrying mobile genetic elements (MGEs) that harbor genes involved in biodegradation of xenobiotic compounds (3, 37). Although it is well known that biodegradation is the main process in the removal of xenobiotic compounds from the environment (3), abiotic factors such as temperature and concentration of pesticides can substantially influence their degradation (4). Accordingly, these abiotic factors might also influence the diversity and abundance of MGEs carrying genes coding for catabolic enzymes.

MGEs such as plasmids, phages, genomic islands, and integrons are often associated with genes coding for enzymes involved in the degradation of xenobiotic compounds and are responsible for the transfer of catabolic genes within bacterial communities (5). Plasmids belonging to the incompatibility groups IncP-1, IncN, IncQ, IncU, and IncW have a broad host range and therefore are of particular interest in studying antibiotic resistance genes or catabolic genes in the environment (6–9). Specifically, IncP-1 plasmids are highly promiscuous plasmids and were reported to carry genes coding for enzymes involved in degradation of different xenobiotic compounds (10–12). Correspondingly, it has been observed that prolonged exposure to pesticides enriches for bacterial populations carrying IncP-1 plasmids (13, 14, 37).

In a PCR-Southern blot-based screening of total community DNA (TC-DNA) from various environments and different geographic locations for the occurrence of potentially catabolic plasmids belonging to the IncP-1, IncP-7, and IncP-9 group, BPS material was revealed as a "hot spot" for these plasmids (15). In accordance with previous findings (14, 37), we hypothesize that temporal changes in the abundance and diversity of MGEs in an on-farm BPS might be caused by changes in the composition and concentration of the pesticides added to the BPS over the course of the agricultural season. To test our hypothesis and to provide the first more comprehensive view on the mobilome of BPS bacterial communities, cultivation-independent techniques were applied to investigate shifts in the abundance and diversity of the MGEs of bacterial communities of a BPS in operation on a farm in Kortrijk, Belgium, over the course of the agricultural season of 2011. During the agricultural season, the BPS material was exposed to different pesticides with various concentrations. The BPS was sam-

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pled three times over the course of the agricultural season, and PCR-based screening of BPS TC-DNA was performed followed by Southern blotting hybridization to check for the presence of broad-host-range, self-transferable and mobilizable plasmids and antibiotic resistance genes often located on class 1 and 2 integrons (*intI1* and *intI2*) and *aadA* gene cassettes. Moreover, the diversity of IncP-1 plasmids was explored by *trfA* amplicon pyrosequencing. Additionally, the concentrations of a range of different pesticides found in the BPS were measured.

MATERIALS AND METHODS

Biopurification system (BPS) material and sampling. Samples were taken from a BPS in Kortrijk, Belgium, containing a biomix composed of coco chips, straw, manure, and field soil. The BPS was used for water contaminated with different types of pesticides (see Table 1) from spillage and residue water collected when cleaning the spraying equipment. The BPS (20 m long, 1.2 m wide) was divided into four compartments, representing four replicates, each of which was sampled three times over the course of the agricultural season of 2011. From each compartment, 12 cores 4 cm in diameter and 10 cm in depth were collected, mixed, dried overnight in a clean bench, and sieved (2 mm pore size). Samples were collected before, during, and after the pesticide spraying season (March, July, and September, respectively). After sieving, the BPS material was stored at 4°C for a few days until TC-DNA extraction. Aliquots of the 12 samples (four replicates from each sampling time) were used for chemical analysis to evaluate the concentrations of pesticides present in the BPS.

Chemical analysis of BPS samples. BPS samples were analyzed for pesticide content by a combination of solid and liquid extraction, cleanup via solid-phase extraction (SPE), and measurement by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as has been proven for several pesticides and their metabolites (16, 17).

In detail, the BPS material was air-dried and thoroughly homogenized. Five g was transferred to ASE (accelerated solvent extraction) cells and extracted sequentially with methanol and acetone/ethyl acetate/water (3:1:1 [vol:vol:vol]). After evaporation of the combined eluates to approximately 3 ml, 250 ml of water was added. The pH was adjusted to 3.8 with acetic acid, and a mixture of the internal reference compounds (diuron-d6, phenazone-d3, and mecoprop [MCPP]-d3 [50 μl, concentrations of 5 ng μl^{-1}]) was added. SPE was performed with Strata X columns (Phenomenex, Torrance, CA) (200 mg, 6 ml) after conditioning with methanol and acidified water. After loading of the samples, cartridges were washed with acidified water, dried for 5 min, and eluted with 5 ml of methanol. The eluate was reduced to approximately 200 μl by a gentle stream of nitrogen, transferred to microinserts of LC vials, and filled with water to reach 1 ml.

Compounds were identified and quantified by LC-MS/MS (Quantum ultra AM mass spectrometer; Thermo Fisher Scientific, Bremen, Germany). Retention times and two characteristic MS/MS transitions were consistent with the reference standards. Separation was performed using a Surveyor-LC system (Thermo Fisher Scientific). It included a quaternary, low-pressure mixing pump with vacuum degassing, an autosampler with a temperature-controlled tray (temperature = 5°C), and a column oven (25°C). The injection volume was 5 μl. For mass spectrometric detection, nitrogen was used as the sheath gas (6 arbitrary units), and helium served as the collision gas. The separations were performed by using a synergy fusion column (Phenomenex, Torrance, CA) (3 μm pore size, 2 by 150 mm) with a H_2O (+10 mM ammonia acetate) (A)/methanol (B) gradient (flow rate, 0.22 ml min^{-1}). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 2 min and linear gradient to 70% A within 22 min and to 100% B after another 3 min. After 100% B isocratic for 9 min, the system returned to its initial condition (90% A) within 1 min and was equilibrated for 6 min. The spectrometer was operated in both positive- and negative-ion modes. Recoveries of the SPE cleanup ranged from 80% to 114% and of the total method, including soil extraction, between 50% and 120%. The limits of quantitation (LOQ) of the target analytes were defined as a signal-to-noise ratio of 10:1 as follows: 2,4-dichlorophenoxyacetic acid (2,4-D), 60 ng g^{-1}; dichlorprop (2,4-DP), 10 ng g^{-1}; amidosulfuron, 1 ng g^{-1}; atrazine, 2 ng g^{-1}; desethyl-atrazine, 30 ng g^{-1}; bromacil, 1 ng g^{-1}; desaminometamitron, 30 ng g^{-1}; metamitron, 10 ng g^{-1}; dimethoate, 10 ng g^{-1}; fenpropidin, 15 ng g^{-1}; ioxynil, 2 ng g^{-1}; isoproturon, 15 ng g^{-1}; 2-methyl-4-chlorophenoxy-acetic acid (MCPA), 20 ng g^{-1}; mecoprop (MCPP), 2 ng g^{-1}; metazachlor, 2 ng g^{-1}; spiroxamine, 10 ng g^{-1}; fluroxypyr, 6 ng g^{-1}; diflufenican, 20 ng g^{-1}; bentazone, 6 ng g^{-1}; metribuzine, 30 ng g^{-1}; fenpropimorph,

TABLE 1 Pesticides added to the BPS[a]

Product name	Active compound(s)
Aramo	Tepraloxydim
Artist	**Flufenacet** **Metribuzin**
Aspect T	**Flufenacet** **Terbuthylazine**
Asulox	Asulam
Atlantis	Iodosulfuron-methyl-sodium Mefenpyr-diethyl Metsulfuron-methyl
Bacara	**Diflufenican** Flurtamone
Beetix	**Metamitron**
Bofix	Clopyralid **Fluroxypyr** 2-Methyl-4-chlorophenoxyacetic acid (MCPA)
Butizyl	4-(4-Chloro-*o*-tolyloxy)butyric acid (MCPB)
Centium	Clomazon
Challenge	Aclonifen
Cycocel	Chlormequat
CIPC	Chlorpropham
Comet	Pyraclostrobin
Defi	Prosulfocarb
Dianal	Fenmedifam
Diquanet	Diquat
Dual Gold	***S*-Metalochlor**
Equip	Foramsulfuron
Ethomat	**Ethofumesate**
Frontier	Dimethenamid-P
Goltix	**Metamitron**
Linuron	Linuron
Metrigon	Clopyralid
MCPA	MCPA*
Mikado	Sulcotrione
Mitron	**Metamitron**
Opus	Epoxiconazole
Pyramin	Chloridazon
Primstar	Florasulam
Primus	Florasulam
Round-Up	Glyphosate
Samson	Nicosulfuron
Starane	**Fluroxypyr**
Stomp	Pendimethalin

[a] List provided by the farmer of Kortrijk, Belgium. Active compounds in bold were detected in the BPS by chemical analysis. *, active compound measured but not detected in the BPS.

TABLE 2 Primer systems for PCR MGE detection and plasmids used for probe generation for Southern blot hybridizations

MGE/antibiotic resistance gene	Gene fragment	Product size (bp)	Primer reference(s)	Reference plasmid(s)
IncP-1	*trfA*	281	23	RP4, R751, pKJK5, pQKH54, pEST4011
IncP-7	*rep*	524	25	pCAR-1
IncP-9	*oriV-rep*	610	15	pM3, pBS2, pBS265, pSN11, pMG18, R2, pNL60, pNL15, pSVS15, pNL22, pNF142
IncQ	*oriV*	436	20, 22	RSF1010
IncN	*repA*	165	20, 22	RN3
IncW	*oriV*	1,140	20, 22	R388
Class 1 integrons	*intI1*	280	26	pKJK5
Class 2 integrons	*intI2*	233	20, 24	pG527
aadA genes[a]			26	pKJK5
sul1		433	21	R388
sul2		293	27	RSF1010

[a] *aadA1*, *aadA2*, *aadA9*, *aadA11*, and *aadA13*.

2 ng g^{-1}; propiconazole, 30 ng g^{-1}; epoxiconazole, 30 ng g^{-1}; tebuconazole, 3 ng g^{-1}; ethofumesate, 20 ng g^{-1}; diuron, 10 ng g^{-1}; terbuthylazine, 2 ng g^{-1}; *S*-metalochlor, 0.1 ng g^{-1}; flufenacetate, 10 ng g^{-1}; and azoxystrobin, 15 ng g^{-1}. External calibration alongside the internal standards was performed at the concentration levels of 10, 25, 50, 200, 5,000, and 2,000 ng ml^{-1} in water/methanol (80:20 [vol:vol]).

Significant differences ($P < 0.05$) in the pesticide concentrations between sampling times were tested by one-way analysis of variance (ANOVA). Measurements were $\log_{10}$ transformed to obtain variance homogeneity verified by Bartlett's test. If statistical significance was found, Tukey's honest significant difference test was used to determine means that are statistically significant from each other. Statistics analyses were done using R 2.15.2 (18).

TC-DNA extraction. Total community DNA (TC-DNA) was extracted from 0.5 g of 2-mm-pore-size-sieved BPS samples by using a FastPrep FP120 bead beating system for cell lysis and a FastDNA Spin kit for soil (MP Biomedicals, Santa Ana, CA) and purified by using a Geneclean Spin kit (MP Biomedicals). The DNA yield and quality were checked by 1% (wt/vol) agarose gel electrophoresis. The absence of PCR-inhibiting substances in the TC-DNA was checked by PCR amplification of 16S rRNA gene fragments using primers and conditions previously described (19) (product size of 1,506 bp).

PCR-Southern blot hybridization detection of IncP-1, IncP-7, IncP-9, IncQ, IncN, and IncW plasmids and *intI1*, *intI2*, *sul1*, *sul2*, and *aadA* genes. The primer systems (20–24) used to detect IncP-1, IncP-7, IncP-9, IncQ, IncN, and IncW plasmids and *intI1*, *intI2*, *sul1*, *sul2*, and *aadA* genes in the TC-DNA and the reference strains used to generate the probes are listed in Table 2. Southern blotting and hybridization were performed as previously described by Binh et al. (22).

Development of a new PCR-Southern blot hybridization system to detect IncU plasmids. A specific probe for Southern blot hybridization detection of IncU plasmids in TC-DNA was generated from the amplified 884-bp fragment of reference plasmid pRA3 using newly designed primers IncU_F3 (5′-TGGCTATCACARGCCGARTT-3′) and IncU_R6 (5′-AGGTTGATCAGNGTGTCRTT-3′) targeting conserved sequences of the replication region of IncU plasmids. Primers were designed based on an alignment of IncU plasmids (Table 3). The PCR mixture (25 μl) contained 1 μl template DNA (1 to 5 ng), 1× *Taq* buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, 0.2 μM (each) primer, and 1.25 U *Taq* polymerase. Initial denaturation was carried out at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. The pRA3 PCR product was analyzed on a 1% (wt/vol) agarose gel, cut from the gel, and purified by using a Qiaex II gel extraction kit. A 10-μl volume of purified PCR product was labeled with digoxigenin (DIG) according to the protocol of the manufacturer (Roche Diagnostics, Mannheim, Germany). The probe was tested for specificity by the absence of cross-hybridization to reference plasmids from IncP-1, IncP-7, IncP-9, IncQ, IncN, and IncW (Table 2). Plasmid pRA3 was used as a positive control for the detection of IncU plasmids in TC-DNA.

IncP-1 *trfA* amplicon pyrosequencing and analysis. The IncP-1 *trfA* gene was sequenced by amplicon pyrosequencing from TC-DNA from all 12 samples. A 281-bp region of the IncP-1 *trfA* gene was amplified using the three primer sets previously described (23) that were also used for Southern blot analysis. Amplification and addition of adapters and barcode tags were done using a U-linker system described by Holmsgaard et al. (28). Briefly, the *trfA* region was PCR amplified using primers with U-linkers attached. Products were purified by agarose gel electrophoresis (29) using a Montage gel extraction kit (Millipore, Billerica, MA). A second round of PCR was used to add sequencing adapters and barcode tags. Approximately equal concentrations of tagged PCR products from all samples were used to ensure nearly equal sample representations. Sequencing was done at The Danish National High-Throughput DNA-Sequencing Centre on a GS FLX Titanium PicoTiterPlate using a GS FLX pyrosequencing system (Roche, Basel, Switzerland).

Primers, U-linkers, barcode tags, and adapters were trimmed away, and sequences were quality filtered and denoised using AmpliconNoise (30). Sequences exceeding 231 ± 3 bp in length were discarded (28). Chimeric sequences were removed *de novo* with uChime in uSearch 5.2.32 (31). In short, sequences were dereplicated and chimeric sequences removed *de novo* based on abundance. These nonchimeric sequences were then used as a reference database to remove chimeric sequences in the standard uChime database mode. The cleaned amplicon sequences were assigned to the seven IncP-1 subgroups by clustering them with known IncP-1 *trfA* reference sequences (see Table S1 in the supplemental material). Sequences from five large clusters, all containing reference sequences, were extracted and independently reclustered at 97% sequence identity. Of the new clusters, only those that contained ≥1% of the total

TABLE 3 Accession number of plasmids used to design primers to detect IncU plasmid-specific amplicons in environment samples

Plasmid	Accession no.
pRA3	DQ401103.1
pMATVIM-7	NC_009739.1
pRSB105	DQ839391.1
pRms149	NC_007100.1
pKBB4037	AJ877266.1
pFBAOT6	NC_006143.1
pHH2–227	JN581942.1
Nitrosomonas eutropha C91 Plasmid2	NC_008342.1
pAb5S9	NC_009476.1
pEIB202	NC_013509.1
Xanthomonas albilineans GPE PC73 plasmII	FP340278.1

TABLE 4 Measurements of pesticide concentrations in the BPS material[c]

Pesticides	LOQ[a]	March	July	September	
Azoxystrobin	15	38 (4.2)	23 (4.5)	24 (5.3)	
Bentazone	6	55,795 (6.0)	53,735 (3.8)	46,060 (1.7)	
Diflufencian	20	671 (2.1)	1,241 (3.7)	1,647 (4.3)	*
Diuron	10	80 (1.2)	108 (2.5)	111 (1.1)	*
Epoxiconazole	30	936 (3.9)	807 (3.6)	612 (4.4)	
Ethofumesate	20	119 (3.9)	136 (5.4)	91 (2.8)	
Fenpropimorph	2	< 4 [b]	5 (4.8)	6 (4.5)	
Fluroxypyr	6	90 (2.8)	1,251 (1.4)	533 (5.9)	*
Flufenacete	10	150 (3.7)	5,062 (1.8)	4,804 (1.9)	*
Metamitron	10	39 (1.9)	63 (1.1)	46 (5.5)	
Metribuzine	30	141 (3.5)	2,065 (5.0)	1,167 (4.5)	*
Propiconazole	30	60 (2.8)	46 (4.3)	168 (4.8)	*
S-metolachlor	10	2,958 (6.0)	3,426 (2.8)	11,806 (1.3)	*
Tebuconazole	3	796 (4.3)	927 (3.5)	941 (2.9)	
Terbuthylazine	2	694 (3.9)	8,579 (2.2)	1,856 (5.6)	*
Total (μg g^{-1})		63 (11.8)	77 (9.1)	70 (8.8)	

[a] LOQ = limit of quantitation.
[b] Three of the four measurements were below the LOQ.
[c] Numbers are means (ng g^{-1}) and percent standard error (SE) ($n = 4$). Darker color indicates higher concentration. *, significant difference between colors (one-way ANOVA, $P < 0.05$).

number of sequences, 27 in total, were used for further analysis. A representative sequence was selected from each new cluster that contained ≥1% of the sequences from the original clusters, and the sequences were aligned using Muscle and used to build a neighbor-joining tree (1,000 bootstrap replicates) in MEGA5.0 (32). Significant differences ($P < 0.05$) in the relative numbers of sequences between sampling times were tested by one-way ANOVA at both clustering levels. Data were $\log_{10}+1$ transformed, and variance homogeneity was verified with Bartlett's test.

Denoising and clustering were done with the QIIME 1.5.0 software package (33) using QIIME tools or QIIME wrappers of other tools. All other bioinformatics analyses were done using Biopieces (www.biopieces.org; developed by Martin A. Hansen), and statistics analyses were done using R 2.15.2 (18).

Nucleotide sequence accession numbers. IncP-1 *trfA* amplicon sequences are submitted to NCBI SRA as study SRA072602 with accession numbers SRS428737, SRS428743, SRS428745, SRS428746, SRS428748, SRS428750, and SRS428751. The IncP-1 *trfA* sequences from the clone library were submitted to GenBank under accession numbers KF576974 to KF576987. See Table 3 for plasmid accession numbers.

RESULTS

Pesticide content of BPS over the course of the agricultural season. Table 4 lists the concentrations of different pesticides found in the on-farm BPS at the three sampling times in March, July, and September. A list of all pesticides applied to the BPS was provided by the farmer from Kortrijk, Belgium (Table 1). However, the concentrations added to the BPS were unknown. Of the 30 pesticides measured by chemical analysis, 15 were not detected: 2,4-dichlorophenoxyacetic acid (2,4-D), dichlorprop (2,4-DP), amidosulfuron, atrazine, bromacil, desaminometamitron, desethylatrazine, dimethoate, fenpropidin, ioxynil, isoproturon, 2-methyl-4-chlorophenoxyacetic acid (MCPA), mecoprop (MCPP), metazachlor, and spiroxamine. Of those, only MCPA was added during the 2011 season. Bentazon (34) and *S*-metalochlor (35), considered recalcitrant to degradation, were found in very high concentrations (μg/g). The concentration of *S*-metalochlor increased from March to September, indicating a slow biodegradation process, if any. However, the concentration of bentazon decreased. Bentazon was not added during the 2011 season; therefore, the bentazon found was probably residual compound from spraying activities in previous seasons. The highest concentrations of about half of the pesticides detected were found in July, and more than half of the pesticides still had a higher concentration in September than initially measured in March. Most of the pesticide compounds that increased in concentration corresponded to compounds added during the 2011 season (see Table 1).

Variations in broad-host-range (BHR) plasmid abundance and diversity in the course of the agricultural season. To investigate the composition and abundance of MGEs in BPS bacteria during the agricultural season, PCR-Southern blot hybridization screenings of BHR and narrow-host-range plasmids belonging to the incompatibility groups IncP-1, IncP-7, IncP-9, IncQ, IncN, IncU, and IncW were performed (Table 5). Strong IncP-1 hybridization signals obtained by using a mixed probe targeting all IncP-1 subgroups indicated that bacterial populations carrying IncP-1 plasmids were very abundant at all sampling times. However, with probes specific for the five different IncP-1 subgroups, a change in the composition of IncP-1 plasmids over the agricultural season was observed. Based on differences in the intensities of hybridization signals detected in the TC-DNA, the abundances of IncP-1β, IncP-1γ, and IncP-1δ plasmids seemed to be affected by the changing concentrations of pesticides. Strong hybridization signals were obtained for the IncP-1β plasmids in March samples, and the signal intensity increased over the year to "very strong" in September. This indicated that bacterial populations carrying IncP-1β plasmids increased in abundance over the year. The signal intensity of IncP-1γ plasmids was weakest in March samples, and the highest hybridization signal was observed in September. There were no changes in the hybridization signal intensities between the three sampling times for IncP-1δ plasmid hybridizations. Strong hybridization signals of IncP-1ε plasmids were observed at all three sampling times, without variation over the season. Strong hybridization signals of IncP-9, IncQ, and IncW plasmids were detected in all samples. IncP-7 plasmids were also detected in all replicates, with a slight increase in the hybridization signal from March to September (Table 5). Hybridization signals of IncU plasmids indicated a gradual decrease in the IncU plasmid abundance from March to September. IncN plasmids were not detected at all, indicating the absence or a low abundance (below Southern blot hybridization detection limits) of these plasmids in the BPS (Table 5).

Variations in IncP-1 plasmid diversity during the course of the agricultural season as revealed by *trfA* amplicon sequencing. The diversity and relative abundances of IncP-1 plasmids were also evaluated by amplicon pyrosequencing of the *trfA* gene region of BPS TC-DNA over the course of the agricultural season. After denoising and chimera removal, 95,199, 49,308, and 57,011 sequences were obtained from primer sets targeting IncP-1αβε, IncP-1γ, and IncP-1δ, respectively. After mixing the sequences from all three primer sets together with 21 known reference sequences from IncP-1 subgroups α, β, γ, δ, ε, and ζ and clustering at 89% sequence identity, 20 clusters were obtained. Eight of these clusters contained the reference sequences along with the majority of amplicon sequences (Fig. 1A). Only 0.03% of the sequences from primer set αβε belonged to the IncP-1α subgroup, and all were from the March samples. No ζ sequences were found, and no sequences clustered with γ pKS208. The clustering level of 89%

TABLE 5 PCR-Southern blot hybridization detection of plasmids in TC-DNA from the BPS[a]

Sample	Hybridization signal strength										
	IncP-1	IncP-1β	IncP-1γ	IncP-1δ	IncP-1ε	IncP-7	IncP-9	IncQ	IncU	IncN	IncW
March (a)	++	+++	++	+++	++++	+++	+++	++++	+	−	++++
March (b)	+++	+++	++	++	++++	++	+++	++++	−	−	++++
March (c)	+++	+++	++	++	++++	++	+++	++++	++	−	++++
March (d)	++	+++	++	+++	++++	++	+++	++++	++	−	++++
July (a)	+++	+++	+++	+++	++++	+++	+++	++++	+	−	++++
July (b)	+++	+++	+++	++	++++	+++	+++	++++	+	−	++++
July (c)	+++	+++	++	++	++++	+++	+++	++++	+	−	++++
July (d)	+++	+++	++	+++	++++	++	+++	++++	+	−	++++
September (a)	+++	++++	+++	+++	++++	+++	+++	++++	+	−	++++
September (b)	+++	++++	+++	++	++++	+++	+++	++++	+	−	++++
September (c)	+++	++++	+++	++	++++	++	+++	++++	+	−	++++
September (d)	+++	++++	+++	+++	++++	+++	+++	++++	+	−	++++
Negative control	−	−	−	−	−	−	−	−	−	−	−
RP4 (IncP-1α)	+++										
R751 (IncP-1β)	+++	+++									
pQKH54 (IncP-1γ)	+++		++++								
pEST4011 (IncP-1δ)	++			+++							
pKJK5 (IncP-1ε)	+++				+++						
pCAR1	+++					+++					
pNF 142							+++				
RSF1010								+++			
pRA3									+++		
RN3										+++	
R388											+++

[a] Hybridization signals: ++++, very strong, with exposure time up to 2 min; +++, strong, with exposure time up to 30 min; ++, strong, with exposure time up to 1 h; +, weak, with exposure time up to 3 h; −, none, with exposure time above 3 h.

was previously determined by Holmsgaard et al. (28). Sequences from the five clusters representing β-1, β-2, γ, δ, and ε and encompassing the large majority of sequences were extracted and reclustered at 97% sequence identity. The 97% value was chosen as a conservative estimate to minimize the effect potential sequencing errors not removed by denoising and chimera checking could have on the estimated plasmid diversity. Pyrosequencing has an error rate of about 1% (36), and the size of the sequences is 237 bp. After reclustering, each original cluster split into many new clusters, with reference sequences found in several of these (Fig. 1B). All β-1 reference sequences were found in cluster 11.101, except pB136, which was found in 11.25. For β-2, almost all amplicon and reference sequences were found in cluster 19.51. Exceptions were pB1 and pAOVO02, which were found in clusters 19.7 and 19.57, respectively. The IncP-1ε sequences split in two major groups, with pKJK5-related plasmids and the large majority of sequences in cluster 18.64 and pEMT3-related plasmids in cluster 18.74. All γ sequences grouped with pQKH54 in cluster 9.3, and no sequences fell into the 9.1 cluster with pMBUI1. Except for the two γ clusters, all representative sequences differed after translation to amino acids. Significant changes in the relative abundances of the different IncP-1 subgroups were found between the three sampling times. IncP1β-1 and -β-2 increased significantly in abundance from March to July (one-way ANOVA, $P < 0.05$) with no change between July and September, whereas IncP-1ε significantly decreased in abundance from March to July and also showed no change between July and September. It is important that the relative abundances were primer specific and cannot be compared among sequences obtained with different primer sets. Furthermore, the numbers of sequences are not related to the abundance of the different IncP-1 subgroups in the TC-DNA samples since the three different primer systems were used for amplicon library construction.

Variations in abundance of integron integrase genes and resistance genes during the course of the agricultural season. The influence of pesticides applied to the BPS on the abundance of integrons and antibiotic resistance genes was investigated by PCR-Southern blot hybridization. Gene-specific sequences of *aadA*, *intI1*, *intI2*, *sul1*, and *sul2* genes were present in all BPS samples. However, while the hybridization signals of *aadA* genes were stable over the season (Table 6), the *intI1*, *intI2*, *sul1*, and *sul2* genes differed in hybridization signal intensities between sampling times. Hybridization signals indicated the highest abundance of *intI2* genes and the lowest abundance of *intI1* in July. The highest hybridization signal of *sul1* antibiotic resistance genes was found in March. A very strong signal indicated a very high abundance of *sul2* resistance genes in March and July, while only weak signals were detected in September.

DISCUSSION

This is the first comprehensive report on the occurrence of various broad- and narrow-host-range plasmids, integrons, and antibiotic resistance genes in on-farm BPS material exposed to different pesticides and monitored with cultivation-independent approaches over an agricultural season. The present study aimed at shedding some light on the mobilome harbored by bacteria in BPS material, as mobile genetic elements are assumed to be important for the adaptation to and biodegradation of pollutants. The present work was motivated by two recent studies on BPS samples from various field sites in Belgium taken at single time points which showed a

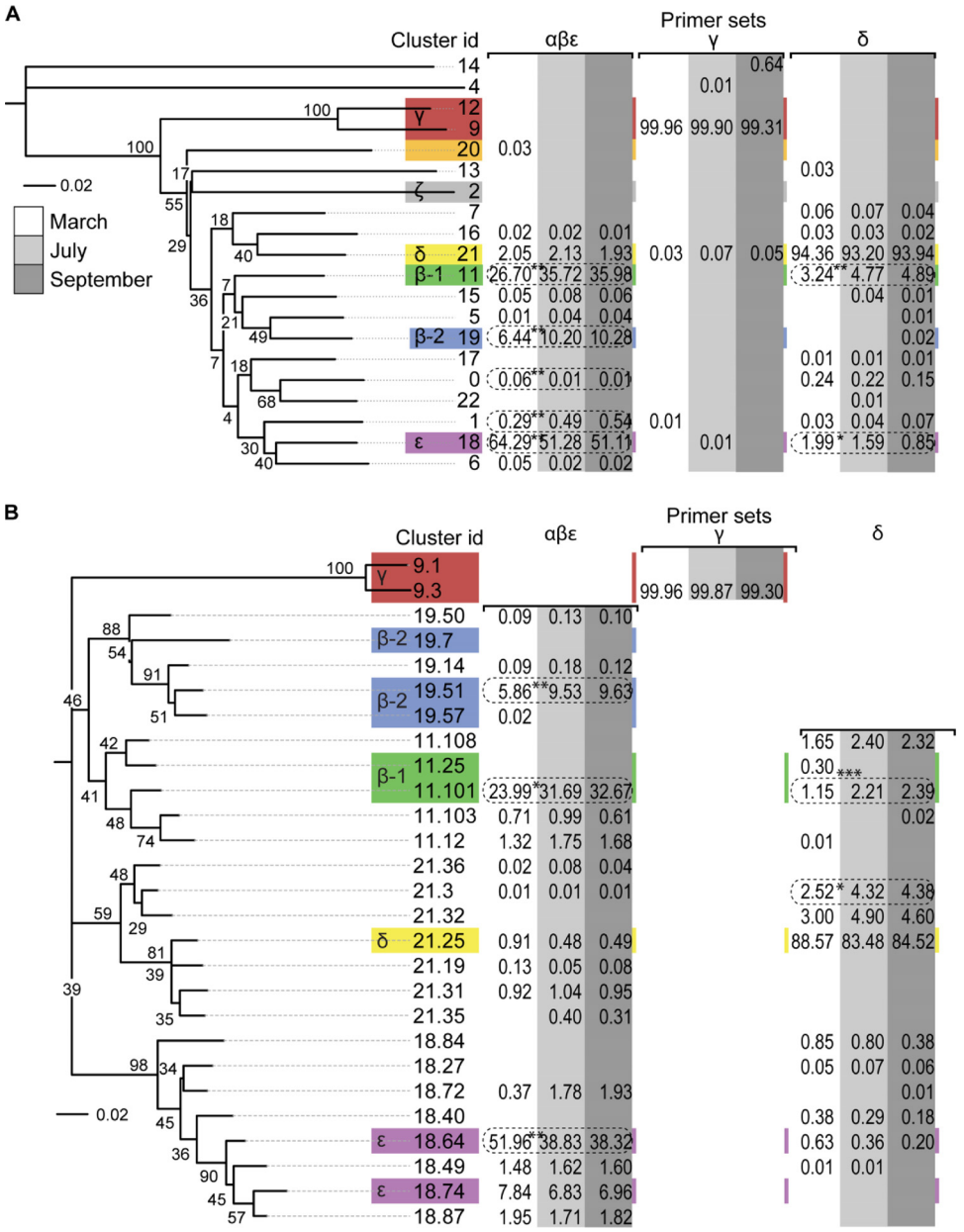

FIG 1 Rooted neighbor-joining trees of clusters of IncP-1 *trfA* amplicon sequences from BPS TC-DNA. Each branch represents one cluster of sequences. Sequences were first clustered at 89% nucleotide sequence identity (A), and sequences from large clusters were then reclustered at 97% similarity (B), with small clusters excluded. Known reference sequences were included in the clustering, and their locations and subgroup names are indicated by colored boxes. The tables show the mean relative abundances of sequences from each primer set. There is a small overlap in targets between primer sets αβε and δ. Dotted circles indicate significant changes in abundance between March, July, and September (one-way ANOVA; $n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Numbers of branches represent percentages of bootstrap support of 1,000 iterations.

high abundance of IncP-1, IncP-7, and IncP-9 plasmids and insertion sequence (IS) elements typically associated with degradative genes (15, 37). Here we monitored fluctuations of a range of different plasmids, integrase genes of class 1 and class 2 integrons, and sulfonamide and streptomycin resistance genes at three times during the agricultural season: before the pesticide spraying season (March), at the height of the spraying season (July), and after the spraying season (September). During the year, the concentrations of different pesticides in the BPS varied considerably and reflected both what the farmers used and the rates of sorption and of biodegradation in the BPS. A semiquantitative but highly sensitive and specific approach was used to detect plasmid replicon-, integrase gene-, and antibiotic resistance gene-specific sequences in TC-DNA. The strong PCR-Southern blot hybridization signals for IncP-1-, IncP-7-, and IncP-9-specific sequences amplified from TC-DNA indicated a high abundance of potentially catabolic plasmids in BPS bacterial communities throughout the agricultural season and thus confirmed previous observations made for single time points from different BPS (15). For the first time, the IncW-, IncQ-, and IncU-specific sequences which typically

TABLE 6 PCR-Southern blot hybridization detection of integrase and antibiotic resistance genes in TC-DNA from the BPS[a]

Sample	Hybridization signal strength				
	intI1	*intI2*	*aadA* genes	*sul1*	*sul2*
March (a)	+++	+	+++	++	++++
March (b)	++++	+	+++	+++	++++
March (c)	++++	+	+++	+++	++++
March (d)	+++	++	+++	−	++++
July (a)	++	++	+++	+	++++
July (b)	++	+++	+++	++	++++
July (c)	++++	+++	+++	++	++++
July (d)	++++	+++	+++	+	++++
September (a)	+++	++	+++	+	++
September (b)	+++	++	+++	+	++
September (c)	+++	++	+++	++	++
September (d)	++	+	+++	+	++
Negative control	−	−	−	−	−
pGT527	−	+	−	−	−
pKJK5	+	+	++	−	−
R388	−	−	−	++	−
RSF1010	−	−	−	−	++

[a] Hybridization signals: ++++, very strong, with exposure time up to 2 min; +++, strong, with exposure time up to 30 min; ++, strong, with exposure time up to 1 h; +, weak, with exposure time up to 3 h; −, none, with exposure time above 3 h.

represent antibiotic resistance plasmids were detected in the BPS TC-DNA for all three sampling times. Interestingly, plasmids belonging to the IncN were below the detection limit. The abundance of IncP-9, IncQ, and IncW plasmids carrying bacterial populations in the BPS seemed to be both very high and rather stable over the season, independently of variations in the concentrations of pesticide present (Table 5). This result indicated that populations carrying these plasmids might not be affected by the different types and concentrations of pesticides added to the BPS over the course of the agricultural season. IncP-9 plasmids might confer an advantage with respect to the survival of the bacterial community in BPS exposed to pesticides or wood-derived polyaromatic hydrocarbons, as IncP-9 plasmids were often found to carry genes encoding different aromatic-ring-degrading enzymes (reviewed in references 6 and 38) which might contribute to the degradation of the pesticides containing an aromatic ring in their chemical structure, such as bentazon, epoxiconazol, and diflufencian.

The very high relative abundances of the promiscuous IncP-1 plasmids detected in the BPS are comparable to those seen with other "hot spots" of horizontal gene transfer such as manure, sewage, or polluted river sediments (39, 40). Our data confirm the results of two recent studies using TC-DNA from different BPS which also found an unusually high abundance of IncP-1 plasmids in the BPS (15, 37). Interestingly, the abundance of IncP-1 plasmids seemed to be increased in BPS bacteria, likely in response to the exposure to different types and concentrations of pesticides as recently reported by Jechalke et al. (14). An increased abundance of IncP-1 plasmids was also observed in microcosm experiments performed with material from the BPS in Kortrijk spiked with linuron or left unspiked (unpublished data). In the microcosm experiment performed with defined exposure to linuron within a rather short time, the abundance of IncP-1 plasmids was increased as revealed by quantitative real-time PCR (S. Dealtry, P. N. Holmsgaard, G.-C. Ding, V. Weichelt, V. Dunon, H. Heuer, L. H. Hansen, S. J. Sørensen, D. Springael, and K. Smalla, unpublished data). Dunon et al. (37) found an increase of IncP-1 plasmid numbers when a BPS matrix was treated with pesticides in a microcosm experiment. Several other studies have pointed to the role of IncP-1 plasmids in the adaptation of the bacterial community to xenobiotic compounds such as pesticides released into the environment (6, 12; reviewed in references 41, 42, and 43). The high abundance of the subgroups IncP-1β, -γ, -δ, and -ε revealed in the present study confirmed previously reported findings (15, 37). Although Southern blot hybridization does not provide quantitative information on specific gene copy numbers per gram of material as does quantitative PCR (qPCR), changes in the abundances of the different subgroups could be indicated which were confirmed by amplicon pyrosequencing of the *trfA* amplicon obtained with the same primer sets as those used for the PCR screening. On the basis of the exposure time of the identical blots hybridized with digoxigenin-labeled probes generated from reference plasmids, we proposed that bacteria carrying IncP-1β and IncP-1ε plasmids were much more abundant in the BPS than those carrying IncP-1α. Similarly, fewer sequences were obtained for IncP-1α plasmids by amplicon pyrosequencing. Different primers were used to target the IncP-1γ and IncP-1δ plasmids; therefore, comparisons should be made only between the IncP-1α, IncP-β, and IncP-1ε groups which were amplified by the same pair of primers. The diversity of different IncP-1 groups in BPS bacterial communities was remarkable and similar to the findings of Bahl et al. (23) for sewage. Hybridizations of *trfA* amplicons revealed an increased abundance of IncP-1β plasmids over the course of the agricultural season, while IncP-1ε levels seemed high and stable. Basic clustering of the *trfA* sequences obtained by pyrosequencing in the different IncP-1 subgroups (Fig. 1A) gave a much more detailed picture of IncP-1 plasmid diversity and also provided insights into the dynamics of bacterial populations hosting these plasmids which likely reflected their exposure to different concentrations of various pesticide compounds. Significant increases in IncP-1β-1 and IncP-1β-2 abundances from March to July were found, and a decrease in IncP-1ε abundance was found in the same period. Interestingly, the Southern blot hybridization of the *trfA* amplicon from TC-DNA indicated an equally high abundance of IncP-1ε plasmids over the season, likely due to the limitations of this method when target sequences are highly abundant. A more detailed clustering of the large subgroups (Fig. 1B) gave no further details on changes in subgroup abundance but showed that almost all sequences had high similarity to those of the well-known IncP-1 plasmids used for reference. Moreover, results obtained by sequencing cloned IncP-1 *trfA* amplicons also indicated that different pesticide concentrations might influence the diversity and distribution of IncP-1 plasmids (see Fig. S1 in the supplemental material).

The high abundance of class 1 and class 2 integrons (*intI1*, *intI2*) and sulfonamide resistance genes (*sul1*, *sul2*) in March and July indicated that BPS are hot spots of the antibiotic resistance genes and integrons which were likely introduced into the BPS bacterial community via manure (Table 6). This could explain why the abundance of *sul1* and *sul2* genes seemed to decrease over the course of the agricultural season (44). In contrast to the bacteria carrying the *intI* and *sul* genes, the bacteria with an aminoglycoside adenylating gene cassette (*aadA*) were stably maintained in the BPS at all sampling times, indicating their occurrence also outside the integron classes investigated here. The *aadA* genes are frequently found on class 1 integrons of environmental isolates

(26); also, the *sul1* genes are associated with class 1 integrons (45), while *sul2* genes are typically found on IncQ plasmids (27, 46). The increased abundance of *intI2* genes in BPS samples taken in July might point to a response of their hosts to pesticides. The addition of manure as a nutrient source before the agricultural season might have also contributed to the high abundances of plasmid groups such as IncQ, IncW, and IncP-1ε, which are typically associated with antibiotic resistance genes and integrons. The application of manure to field soil is known to introduce not only antibiotic compounds and their metabolites but also bacteria carrying plasmids, integrons, and antibiotic resistance genes (reviewed in reference 39).

To the best of our knowledge, this is the first comprehensive report on the occurrence of a range of different MGEs in an on-farm BPS over the course of an agricultural season. Here we showed that on-farm BPS might be considered another hot spot of mobile genetic elements and horizontal gene transfer (40). The exposure of BPS bacteria to various pollutants might have fostered the adaptation to rapidly changing environmental conditions via horizontally acquired mobile genetic elements. In particular, the amplicon sequencing results revealed not only an impressive diversity of IncP-1 plasmids but also a dynamic bacterial population carrying IncP-1β and IncP-1ε plasmids over the course of an agricultural season. The present study showed that bacterial populations carrying IncP-1β plasmids were increased in abundance (positively affected) by the exposure of the on-farm BPS to various pesticides. The increased abundance of IncP-1β plasmids might result from growth of existing populations or from horizontal transfer of IncP-1β plasmids to other host bacteria. The results strongly indicated that the bacterial populations carrying IncP-1β plasmids might play a major role in pesticide degradation in such polluted systems.

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